EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
**COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED**

# Sampling and DNA Extraction of sugar beet H7-1

# Report from the Validation of the "CTAB Precipitation/Genomic-tip" method for DNA extraction from ground sugar beet seeds

**Method development and single laboratory validation:**
KWS SAAT AG and Monsanto Company

**Method testing and confirmation:**
Joint Research Centre – European Commission
Biotechnology & GMOs Unit

# Contents

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# 1. Introduction

Purpose of the DNA extraction procedure described is to serve as a method to provide DNA for subsequent PCR-based detection methods. The method does not only have to yield DNA of sufficient quality and quantity but is also required to be suitable for routine use in terms of ease of operations, sample throughput and costs. This Report describes the method and validation experiments including results.

# 2. Description of the methods

**Sampling:**

For details concerning requirements of particle size/particle number, official ISTA-guidelines (International Rules for Seed Testing, 1999, Volume 27, Supplement, Rules, 1999.) and/or sampling according to prCEN/TS 21568 (2005 - Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Sampling strategies) are proposed .

**Scope and applicability:**

The "CTAB Precipitation/Genomic-tip" method for DNA extraction described below is suitable for the isolation of genomic DNA from a wide variety of matrices. However, validation data presented here are restricted to ground sugar beet seeds. Application of the method to other matrices may require adaptation and needs specific validation.

**Principle:**

The basic principle of DNA extraction consists of first releasing the DNA present in the matrix into aqueous solution and further, concurrently or subsequently, purifying the DNA from PCR inhibitors. The "CTAB Precipitation/Genomic-tip" method starts with a lysis step (thermal lysis in the presence of CTAB, EDTA, RNase A and Proteinase K) followed by removal of contaminants such as lipophilic molecules and proteins by extraction with chloroform. Afterwards a DNA precipitate is generated by using CTAB precipitation buffer (under low salt conditions DNA precipitates in the presence of CTAB). The pellet is

dissolved in TE-buffer. Afterwards, remaining inhibitors are removed by an anion exchange chromatography step using Genomic-tip 20 columns (QIAGEN).

**Note:**

If starting from intact sugar beet seed samples instead of finely ground sugar beet seed samples, a grinding device such as a laboratory mill or blender has to be used. Milling/grinding of grain/seed not only facilitates the lysis by mechanically disrupting cellular structures and increasing the surface area, but is also indispensable for the generation of representative test portions by reducing the particle size. For details concerning requirements of particle size/particle number, generation of the test portions etc. please refer to the literature (e. g. prCEN/TS ISO 21568 sampling).

DNA extractions are recommended to be carried out at least on two test portions. Extraction blanks in duplicate (negative controls; handled identically but without sample material) are mandatory throughout extraction and subsequent PCR.

**Lysis / CTAB precipitation of DNA**

1. Transfer 10 ml CTAB buffer and 40 µl Proteinase K and 10 µl of Rnase A to 50 ml conical tube.
2. Weigh out 2 g of ground sugar beet into the tube containing CTAB buffer, Proteinase K and RNase A, mix thoroughly.
3. Incubate overnight at 60°C with agitation.
4. Allow the lysate to cool down to room temperature (approx. 15 min) on the bench.
5. Add 5 ml of chloroform and mix completely by inverting the tubes for 5 min (by hand or by using an overhead mixer).
6. Spin down at room temperature for 5 minutes at 10000 x *g*.
7. Transfer 7.5 ml of the supernatant to a new conical tube and add 15 ml CTAB precipitation buffer and mix completely.
8. Incubate for 60 minutes at room temperature.
9. Centrifuge at room temperature for 20 minutes at approximately 10000 x *g*.
10. Discard supernatant completely.

11. Dissolve the pellet in 1 ml 1x TE (pre-warmed to 50 °C). Let stand for 15 min at room temperature with occasional mixing. Make sure that the pellet is dissolved completely.

**Purification using QIAGEN Genomic-tip 20/G columns and QIAGEN DNA buffer set**

12. Add 9.5 ml G2-buffer, 10 µl Proteinase K and 10 µl RNase A and mix completely.
13. Incubate for 60 minutes at 50 °C with agitation.
14. Centrifuge at room temperature for 5 minutes at 10000x *g*.
15. In the meantime equilibrate each QIAGEN Genomic-tip 20/G with 2 ml buffer QBT.
16. Transfer the clear supernatant to the equilibrate Genomic-tip 20/G and allow it to enter the resin by gravity flow.
17. Wash the column with 3x1 ml buffer QC.
18. Elute the genomic DNA with 2x1 ml buffer QF (pre-warmed to 50 °C. Collect each of the 1 ml eluate separately in a 2 ml microcentrifuge tube.
19. Add 700 µl isopropanol to each eluate in the 2 ml microcentrifuge tubes and invert the tubes 10-20 times.
20. Centrifuge at 4 °C for 15 minutes at approximately 20000x *g*.
21. Discard supernatant, add 1 ml 70% ethanol and mix completely (e.g. vortex briefly).
22. Centrifuge at 4 °C for 10 minutes at approximately 20000x *g.*
23. Carefully remove and discard the supernatant. Centrifuge again and remove all remaining ethanol. If any fluid remains, allow pellet to dry at room temperature.
24. Re-suspend the pellet in 150 µl 1 x TE buffer. Let stand for 15 min at room temperature with occasional mixing. Make sure that the pellet is dissolved, than centrifuge for 2 minutes at approximately 20000x *g* and combine the supernatant of the same sample into a fresh 1.5 ml microcentrifuge tube (final volume for each sample extract: 300 µl).

# 3. Equipment / Reagents / Plasticware

## 3.1. Equipment

The following equipment is used in the DNA extraction procedure described (equivalents may be substituted):

| **Equipment** | **Example of appropriate apparatus** |
|---|---|
| Pipettes with adjustable volume | e. g. Eppendorf Research. 2 – 20 µl. 20 – 200 µl. 100 -1000 µl |
| Incubator with shaker or shaking water bath | e. g. Heraeus Function Line B12 in combination with shaker GFL 3005 (Gesellschaft für Labortechnik mbH) |
| Balances for the preparation of buffers and solutions and for sample weigh in | e. g. Ohaus Scout II. Ohaus Adventurer |
| Centrifuge with rotors for 50 ml centrifuge tubes and microcentrifuge tubes | e. g. Sigma 4 K 15C with suitable rotors |
| Thermoblock for 1,5 / 2,0ml microcentrifuge tubes | e. g. Bioblock Scientific 92333 |
| Vortex | e. g. NeoLab Vortex VM-300 |
| Overhead | e.g. Heidolph REAX 2 |

## 3.2. Reagents

The following reagents are used in the DNA extraction procedure described (equivalents may be substituted):

| **Reagent** | **Specification** |
|---|---|
| NaCl | p. a. quality or Molecular biology grade |
| CTAB | p. a. quality or Molecular biology grade |
| Tris | p. a. quality or Molecular biology grade |
| EDTA · $Na_2$-salt | p. a. quality or Molecular biology grade |
| HCl | p. a. quality |
| Proteinase K | from Tritirachium album, DNAses, Rnases, Exonucleases not detectable, Molecular biology grade |
| RNase A | from bovine pancreas, salt free, protease free and chromatographically homogeneous, ca. 90 Kunitz units/mg |
| Sodium acetate | p. a. quality or Molecular biology grade |
| Chloroform | p. a. quality |
| Isopropanol | p. a. quality |
| Ethanol | p. a. quality |
| QIAGEN Genomic DNA buffer set including buffers G2, QBT, QC and QF | Cat. no: 19060, QIAGEN, Germany |
| QIAGEN Genomic-tip 20/G | Cat. no: 10223, QIAGEN, Germany |

The following buffers and solutions are used in the DNA extraction procedure described:

**CTAB buffer**

1,4 M NaCl

2% (w/v) CTAB

0,1 M Tris-Base

0,015 M EDTA

For 1 litre CTAB buffer weigh out 81,8 g NaCl. 20 g CTAB. 12,1 g Tris-Base and 5,84 g EDTA in an appropriate beaker and add about 800 ml $H_2O_{deion}$. Adjust pH with HCl to pH 8,0, stir until all reagents are dissolved. Adjust volume to 1 litre with $H_2O_{deion}$. Do not autoclave.

Store at room temperature for up to 2 years.

**CTAB precipitation buffer**

40 mM NaCl

0.5% (w/v) CTAB

For 1 litre CTAB precipitation buffer weigh out 2,34 g NaCl and 5 g CTAB in an appropriate beaker. Adjust volume to 1l with $H_2O_{deion}$ and stir until all reagents are dissolved. Do not autoclave.

Store at room temperature for up to 1 year.

**Proteinase K**

20 mg/ml $H_2O$

For 10 ml Proteinase K solution dissolve 200 mg Proteinase K in 10ml $H_2O_{deion}$,

Store at -20°C for up to 2 years.

**RNase A**

91 mg/ml

Dissolve 0,5 g RNase A in 5 ml 0,01 M sodium acetate (pH 5,2). Aliquot in 1ml portions, boil for 15 minutes to inactivate DNases, cool slowly to room temperature and add 100 µl 1M Tris-HCl (pH 7,4) to each aliquot. Store at -20°C for up to 2 years.

**70% (v/v) Ethanol**

For 200 ml combine 140 ml 100% ethanol and adjust volume to 200 ml $H_2O_{deion}$.

Store at room temperature for up to 5 years.

**1x TE buffer**

10 mM Tris. pH 8,3

1 mM EDTA

For 100 ml 1x TE buffer combine 1 ml 1M Tris (pH 8,3) and 200 µl 0,5 M EDTA (pH 8,0) and adjust the volume to 100 ml with $H_2O_{deion}$. Autoclave.

Store at room temperature for up to 2 years.

**0,2x TE buffer**

2 mM Tris, pH 8,3

0,2 mM EDTA

For 100 ml 0,2x TE buffer dilute 20 ml 1x TE buffer with 80 ml $H_2O_{deion}$. Autoclave.

Store at room temperature for up to 2 years.

**G2, QBT, QC and QF buffer**

Are included in the QIAGEN Genomic DNA buffer set (Cat n.: 19060).

Preparation of these buffers is described in the manufacturer's instructions.

## 3.3. Plasticware

Note: All plastic ware has to be sterile and free of DNases, RNases and nucleic acids.

| **Item** | **Specification** |
|---|---|
| 50 ml conical tubes | e. g. Sarstedt, 62.547 254 |
| 1,5 ml microcentrifuge tube | e. g. Roth, 4182.1 |
| 2 ml microcentrifuge tube | e. g. Eppendorf, 30.120.094 |
| filter tips | fitting the pipette models used |

# 4. Experimental Validation and Results

For experimental validation six different samples of ground sugar beet seeds were extracted using the above described method. Five samples were extracted in duplicate, one sample was extracted sixfold.

The following performance characteristics were determined:

- DNA concentration / yield. Repeatability
- Fragmentation state of DNA
- Purity / absence of PCR-inhibitors

## 4.1. DNA concentration / Yield, Repeatability

The concentration of the DNA extracts was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). DNA extracts were diluted (in triplicates) in 0.2x TE buffer and were mixed in a 1:2 ratio with PicoGreen reagent. DNA concentration was determined on the basis of a five-point standard curve (triplicates) ranging from 10 ng/µl to 500 ng/µl using an ABI-PRISM® 7700 as a detector. Sixfold extraction of one sample was the prerequisite for calculation of statistical parameters average, standard deviation and coefficient of variation.

| Sample | Replicate | Concentration [ng/µl] |
|---|---|---|
| 1 | i | 118 |
| | ii | 137 |
| 2 | i | 78 |
| | ii | 96 |
| 3 | i | 63 |
| | ii | 62 |
| 4 | i | 145 |
| | ii | 151 |
| 5 | i | 61 |
| | ii | 63 |
| 6 | i | 113 |
| | ii | 126 |
| | iii | 111 |
| | iv | 104 |
| | v | 102 |
| | vi | 114 |

**DNA concentration (ng/µl):**

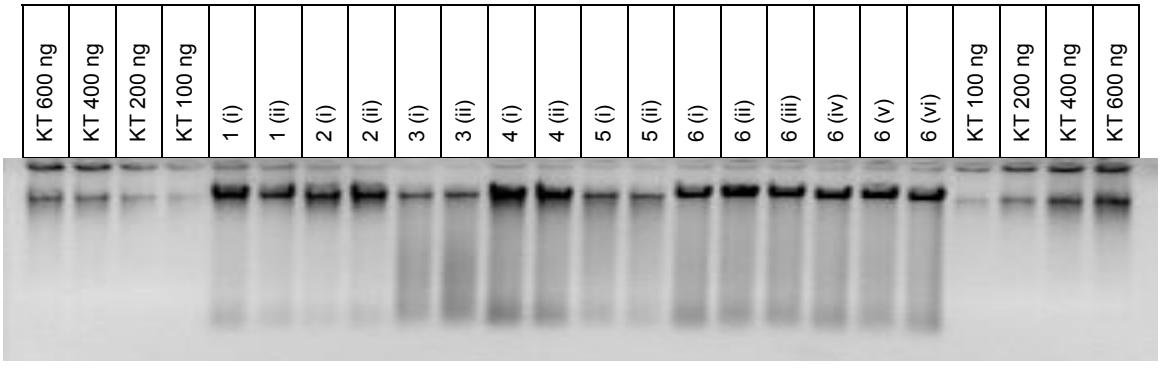
| | |
|---|---|
| Overall average of all samples: | 103 ng/µl |
| Average (sample no. 6, sixfold extraction): | 112 ng/µl |
| Standard deviation (sample no. 6, sixfold extraction): | 8.5 ng/µl |
| Coefficient of variation (sample no. 6, sixfold extraction): | 7.6% |

**Yield (total volume of DNA solution: 300 µl):**

| | |
|---|---|
| Overall average of all samples: | 30.8 µg |
| Average (sample no. 6, sixfold extraction): | 33.5 µg |
| Standard deviation (sample no. 6, sixfold extraction): | 2.6 µg |
| Coefficient of variation (sample no. 6, sixfold extraction): | 7.6% |

## 4.2. Fragmentation state of DNA

The fragmentation state of the extracted DNA was evaluated by agarose gel electrophoresis: 5 µl of each DNA solution was analyzed on a 1,5% agarose gel (TAE buffer system). Defined amounts of calf thymus DNA (KT) were loaded as DNA quantity standards. After electrophoretic separation the gel was stained in ethidium bromide solution for 15 min and the DNA visualized using an UV transilluminator.




Medium to high molecular weight DNA was observed.

## 4.3. Purity / absence of PCR-inhibitors

In order to assess the PCR quality of the extracted DNAs, the samples were tested with a sugar beet specific real–time PCR system (reference system "GS") for the presence of inhibitory substances. For this purpose four independent serial dilutions (fourfold 1:4, 1:16, 1:64, 1:256) of the 40 ng/µl stocks of each sample with 0.2 x TE buffer were prepared. To measure inhibition, the Ct values of the four diluted samples were plotted against the natural logarithm of the dilution and the Ct value for the undiluted sample was extrapolated from the equation calculated by linear regression. Subsequently the extrapolated Ct for the undiluted sample was compared with the measured Ct. Evaluation: PCR inhibitor is present if the measured Ct value for the undiluted sample is suppressed by > 0,5 cycles from the calculated Ct value.

**Ct values of undiluted and fourfold serial diluted DNA extracts:**

| | undiluted | diluted | | | |
|---|---|---|---|---|---|
| **DNA extract** | **1:1** | **1:4** | **1:16** | **1:64** | **1:256** |
| **1 (i)** | 20,05 | 21,84 | 23,87 | 26,04 | 28,31 |
| **1 (ii)** | 20,22 | 22,40 | 24,08 | 25,67 | 27,81 |
| **2 (i)** | 20,76 | 22,49 | 24,55 | 26,71 | 28,69 |
| **2 (ii)** | 20,34 | 22,34 | 24,58 | 26,88 | 28,55 |
| **3 (i)** | 19,72 | 22,09 | 24,00 | 26,32 | 28,06 |
| **3 (ii)** | 19,60 | 21,70 | 23,70 | 25,78 | 27,77 |
| **4 (i)** | 19,57 | 21,91 | 23,89 | 26,14 | 28,20 |
| **4 (ii)** | 20,00 | 22,29 | 24,10 | 26,56 | 28,39 |
| **5 (i)** | 19,99 | 22,16 | 24,27 | 27,11 | 28,66 |
| **5 (ii)** | 19,95 | 21,83 | 24,27 | 26,77 | 28,75 |
| **6 (i)** | 19,82 | 21,78 | 23,99 | 26,21 | 28,02 |
| **6 (ii)** | 20,08 | 22,10 | 24,09 | 26,43 | 28,28 |
| **6 (iii)** | 19,68 | 21,85 | 24,15 | 25,98 | 28,14 |
| **6 (iv)** | 19,69 | 21,72 | 24,05 | 26,01 | 28,05 |
| **6 (v)** | 19,93 | 21,87 | 24,05 | 26,04 | 28,32 |
| **6 (vi)** | 19,78 | 22,07 | 24,11 | 26,29 | 28,21 |

## Comparison of extrapolated Ct values versus measured Ct values:

**delta Ct = abs (Ct extrapolated - Ct measured)**

| DNA extract | Ct extrapolated | $C_T$ measured | delta Ct |
|---|---|---|---|
| **1 (i)** | 19,62 | 20,05 | 0,43 |
| **1 (ii)** | 20,54 | 20,22 | 0,32 |
| **2 (i)** | 20,42 | 20,76 | 0,34 |
| **2 (ii)** | 20,36 | 20,34 | 0,02 |
| **3 (i)** | 19,67 | 19,60 | 0,07 |
| **3 (ii)** | 19,76 | 19,57 | 0,19 |
| **4 (i)** | 20,15 | 20,00 | 0,15 |
| **4 (ii)** | 19,97 | 19,99 | 0,02 |
| **5 (i)** | 19,59 | 19,95 | 0,36 |
| **5 (ii)** | 20,21 | 19,99 | 0,22 |
| **6 (i)** | 19,77 | 19,82 | 0,05 |
| **6 (ii)** | 20,01 | 20,08 | 0,07 |
| **6 (iii)** | 19,86 | 19,68 | 0,18 |
| **6 (iv)** | 19,72 | 19,69 | 0,03 |
| **6 (v)** | 19,74 | 19,93 | 0,19 |
| **6 (vi)** | 20,02 | 19,78 | 0,24 |

All delta Ct values of extrapolated versus measured Ct are < 0,5. The data observed indicate absence of PCR inhibitors.

# 5. Experimental testing of the DNA extraction method by the Community Reference Laboratory

The aim of the experimental testing is to verify that the method of DNA extraction provides DNA of suitable quantity and quality for the intended purpose.
The DNA extraction method should allow preparation of the analyte in amounts and quality appropriate for the analytical method used to quantify the event-specific analyte versus the reference analyte.
The CRL tested the CTAB Precipitation/Genomic-tip DNA extraction method proposed by the applicant on the H7-1 sugar beet line. To assess the suitability of the extraction method for Real Time PCR analysis, the DNA extracted was tested using a qualitative PCR run on the real time PCR equipment.
The experimental testing was carried out on H7-1 ground sugar beet seeds provided by the applicant.

## 5.1 Preparation of samples

About 90 g of the transgenic H7-1 sugar beet seed material were grinded using a GRINDOMIX mixer.

## 5.1 DNA extraction

DNA was extracted by means of the CTAB Precipitation/Genomic-tip 20G method described above and in-house validated by the applicant.
The DNA extraction was carried out on 6 test portions (replicates) and repeated in three different days, giving a total of 18 DNA extracts.

**Note**: the following modification to the method was introduced: step n.12, 100 µl of Proteinase K (20 mg/ml) were added instead of 10 µl as indicated in the original protocol, following the instruction given in the QIAGEN Genomic DNA Handbook for mini tip 20/G.

## 5.2 DNA concentration / Yield, Repeatability

DNA concentration of the DNA extracted was determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes).

Each DNA extract was measured twice, and the two values were averaged. DNA concentration was determined on the basis of a five point standard curve ranging from 1 to 500 ng/µl using a Biorad VersaFluor fluorometer.

Considering that the expected DNA concentration of the samples was close to the upper limit of the range, samples were diluted 1:1 prior to measurement.

The DNA quantification for all samples (yellow boxes samples from 1 to 6 extracted on 08.03.05, green boxes samples from 1-6 extracted on 09.03.05 and blue boxes samples extracted on 16.03.05) is reported in the table below.

| Sample | Concentration (ng/µl) |
|---|---|
| **1** | 13 |
| **2** | 182 |
| **3** | 150 |
| **4** | 11 |
| **5** | 134 |
| **6** | 183 |
| **1** | 158 |
| **2** | 98 |
| **3** | 115 |
| **4** | 126 |
| **5** | 126 |
| **6** | 128 |
| **1** | 172 |
| **2** | 170 |
| **3** | 154 |
| **4** | 164 |
| **5** | 333 |
| **6** | 214 |

**DNA concentration (ng/µl)**

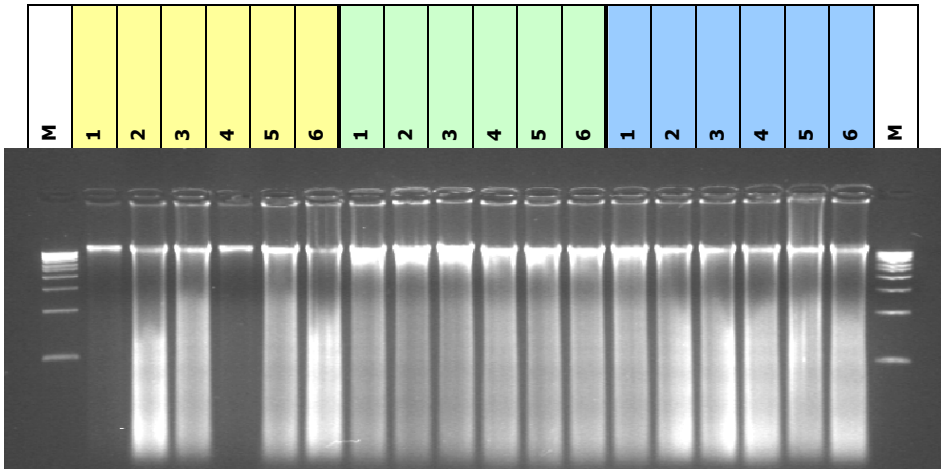
| | |
|---|---|
| Overall average of all samples: | 146.3 ng/µl |
| Standard deviation of all samples | 70.5 ng/µl |
| Coefficient of variation | 48.2% |

**Yield (total volume of DNA solution: 300 μl)**

| | |
|---|---|
| Overall average of all samples: | 43.9 μg |
| Standard deviation | 21.2 μg |
| Coefficient of variation | 48.2.2% |

## 5.3 Fragmentation state of DNA

The size of the extracted DNA was evaluated by agarose gel electrophoresis; 5 µl of the DNA solution were analyzed on a 1.5% agarose gel. In yellow boxes samples from 1 to 6 extracted on 08.03.05; in the green boxes samples from 1-6 extracted on 09.03.05; in the blue boxes samples extracted on 16.03.05. A DNA ladder 1kb (M) was used.




Low and high molecular weight DNA distribution was observed.

## 5.4 Purity / Absence of PCR inhibitors

To assess the PCR quality of the DNA extracted, the experimental approach previously described (see paragraph 4.3) was followed.
Yellow boxes samples from 1 to 6 extracted on 08.03.05, green boxes samples from 1-6 extracted on 09.03.05 and blue boxes samples extracted on 16.03.05).

### Ct values of undiluted and fourfold serial diluted DNA extracts

| | Undiluted (40 ng/µl) | diluted | | | |
|---|---|---|---|---|---|
| **DNA extract** | **1:1** | **1:4** | **1:16** | **1:64** | **1:256** |
| **1** | n.a. | n.a. | n.a. | n.a. | n.a. |
| **2** | 18,49 | 20,50 | 22,64 | 24,64 | 26,91 |
| **3** | 18,74 | 20,74 | 22,88 | 25,24 | 27,30 |
| **4** | n.a. | n.a. | n.a. | n.a. | n.a. |
| **5** | 18,79 | 20,97 | 23,00 | 25,19 | 27,00 |
| **6** | 18,79 | 20,94 | 23.14 | 25,32 | 27,43 |
| **1** | 19,23 | 21,32 | 23,18 | 25,41 | 27,23 |
| **2** | 18,229 | 20,43 | 22,53 | 24,77 | 26,33 |
| **3** | 18,40 | 20,36 | 22,56 | 24,63 | 26,54 |
| **4** | 18,78 | 20,73 | 22,73 | 25,12 | 27,10 |
| **5** | 18,76 | 20,74 | 22,89 | 24,80 | 27,05 |
| **6** | 17,66 | 19,65 | 21,72 | 23,79 | 25,77 |
| **1** | 18,73 | 20,51 | 22,49 | 24,58 | 26,64 |
| **2** | 18,97 | 20,74 | 22,98 | 24,90 | 26,74 |
| **3** | 18,65 | 20,62 | 22,64 | 24,75 | 26,78 |
| **4** | 18,93 | 20,92 | 22,92 | 25,04 | 27,21 |
| **5** | 19,76 | 21,70 | 23,86 | 25,88 | 27,91 |
| **6** | 19,37 | 21,13 | 23,18 | 25,22 | 27,36 |

Note: the PCR quality of the samples number 1 and 4 in the yellow boxes (n.a.), was not assessed due to the low DNA yield obtained after extraction. However it cannot be asserted that the DNA of these samples is not suitable for real-time analysis.

**Comparison of extrapolated Ct values versus measured Ct values:**

| DNA extract | $R^2$ | Slope* | Ct extrapolated | $C_T$ measured | delta Ct** |
|---|---|---|---|---|---|
| **1** | - | - | - | - | - |
| **2** | 0,9923 | -3,48 | 18,36 | 18,49 | 0,13 |
| **3** | 0,9961 | -3,58 | 18,53 | 18,74 | 0,21 |
| **4** | - | - | - | - | - |
| **5** | 0,9964 | -3,41 | 18,96 | 18,79 | -0,17 |
| **6** | 0,9978 | -3.59 | 18,79 | 18,79 | 0 |
| **1** | 0,9977 | -3,33 | 19,29 | 19,24 | -0,05 |
| **2** | 0,9927 | -3,39 | 18,53 | 18,29 | -0,24 |
| **3** | 0,9982 | -3,41 | 18,37 | 18,40 | 0,03 |
| **4** | 0,9977 | -3,49 | 18,54 | 18,78 | 0,24 |
| **5** | 0,9972 | -3,43 | 18,66 | 18,76 | 0,10 |
| **6** | 0,9962 | -3,37 | 17,64 | 17,66 | 0,02 |
| **1** | 0,9984 | -3,31 | 18,43 | 18,72 | 0,29 |
| **2** | 0,9976 | -3,27 | 18,86 | 18,97 | 0,11 |
| **3** | 0,9964 | -3,39 | 18,55 | 18,65 | 0,10 |
| **4** | 0,9983 | -3,43 | 18,78 | 18,93 | 0,15 |
| **5** | 0,9981 | -3,40 | 19,68 | 19,76 | 0,08 |
| **6** | 0,9994 | -3,33 | 19,03 | 19,37 | 0,34 |

Note: In the yellow boxes samples from 1 to 6 extracted on 08.03.05; in the green boxes samples from 1-6 extracted on 09.03.05

***The expected Slope for a PCR with 100% efficiency is -3.32**
****delta Ct = abs (Ct extrapolated - Ct measured)**

All delta Ct values of extrapolated versus measured Ct are < 0,5

# 6. Conclusion

The data reported show that the method is fit for the intended purpose.

# 7. Literature

Sambrook. J., Fritsch. E. F. and Maniatis. T. (1989) Molecular Cloning: a laboratory manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

# 8. Abbreviations

| | |
|---|---|
| CTAB | cetyltrimethylammoniumbromide |
| EDTA | ethylenediaminetetraacetic acid |
| KT | calf thymus |
| PCR | polymerase chain reaction |
| RNase A | ribonuclease A |
| TAE | tris-acetate |
| TE | tris EDTA |
| Tris | tris(hydroxymethyl)aminomethane |